REGULAR ARTICLE

blood advances

The bacterial microbiota regulates normal hematopoiesis via metabolite-induced type 1 interferon signaling

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Key Points

- The intestinal microbiota promotes hematopoiesis by activating type I IFN and STAT1 signaling.
- Metabolites of the intestinal microbiota such as NOD1 ligand can rescue hematopoiesis in antibiotic-treated mice.

Antibiotic therapy, especially when administered long term, is associated with adverse hematologic effects such as cytopenia. Signals from the intestinal microbiota are critical to maintain normal hematopoiesis, and antibiotics can cause bone marrow suppression through depletion of the microbiota. We reported previously that STAT1 signaling is necessary for microbiota-dependent hematopoiesis, but the precise mechanisms by which the gut microbiota signals to the host bone marrow to regulate hematopoiesis remain undefined. We sought to identify the cell type(s) through which STAT1 promotes microbiota-mediated hematopoiesis and to elucidate which upstream signaling pathways trigger STAT1 signaling. Using conditional knockout and chimeric mice, we found that the microbiota induced STAT1 signaling in non-myeloid hematopoietic cells to support hematopoiesis and that STAT1 signaling was specifically dependent on type I interferons (IFNs). Indeed, basal type I IFN signaling was reduced in hematopoietic progenitor cells with antibiotic treatment. In addition, we discovered that oral administration of a commensal-derived product, NOD1 ligand, rescues the hematopoietic defects induced by antibiotics in mice. Using metabolomics, we identified additional microbially produced candidates that can stimulate type I IFN signaling to potentially rescue the hematopoietic defects induced by antibiotics, including phosphatidylcholine and γ-glutamylalanine. Overall, our studies define a signaling pathway through which microbiota promotes normal hematopoiesis and identify microbial metabolites that may serve as therapeutic agents to ameliorate antibiotic-induced bone marrow suppression and cytopenia.

Introduction

Although antibiotic therapy can be lifesaving in the context of serious bacterial infections, it is associated with a variety of adverse effects, especially when administered long term.[1] Cytopenias, such as neutropenia, anemia, or thrombocytopenia, are highly prevalent adverse outcomes of prolonged antibiotic administration. Up to 20% of individuals receiving antibiotics report one or more cytopenias, although the prevalence varies depending on agent and duration of therapy.[2-4]

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Metabolomics data will be made publicly available through Mendeley data sharing (https://data.mendeley.com/datasets/63nd69rbv9/1).

The full-text version of this article contains a data supplement.

Antibiotic-associated bone marrow suppression is an important clinical problem that interferes with treatment and inflates health care costs.[5] Antibiotics are often used to prevent or treat febrile neutropenia after hematopoietic stem cell transplantation, but loss of microbial diversity in these patients has been linked to increased risk of transplant-related death and poorer overall survival due to graft-versus-host disease, poor engraftment, and increased late-stage infections.[6-9] In mice, studies have shown that antibiotic-treated mice in standard specific pathogen–free (SPF) housing conditions have defects in systemic immunity, as antibiotic-driven loss of progenitor populations decreases peripheral immune cells, increasing susceptibility to systemic infections.[10,11]

Antibiotic-associated bone marrow suppression is observed with many antibiotics, including β-lactam antibiotics,[12] cephalosporins[13,14] such as ceftaroline,[3,15] and linezolid.[2] Initially, these hematologic side effects were presumed to be due to direct action of antibiotics on hematopoietic cells. However, research by our group and others has both shown both a lack of direct antibiotic-mediated cytotoxicity for hematopoietic progenitor populations[16,17] and identified indirect mechanisms of antibiotic-mediated bone marrow suppression.[18] Specifically, antibiotics have been shown to disrupt healthy steady-state hematopoiesis by suppressing microbial populations in the intestine.[5,10,19-21]

The complex community of gut microbes exerts a plethora of local and systemic effects on host immunity, including regulating the development and maturation of immune cells.[22] Germ-free mice, which lack a microbiota, have defective immune systems and exhibit both altered hematopoietic environments and decreased progenitor cells in the bone marrow.[10,16] Importantly, antibiotic therapy administered to conventional, SPF mice similarly alters the hematopoietic compartment, causing loss of bone marrow cellularity and depleting hematopoietic stem and progenitor cells (HSPCs) as well as granulocyte populations.[16,19,21] Our prior work found that these factors are mechanistically linked; antibiotic treatment of germ-free mice does not further suppress their blood cell production, whereas replenishing the intestinal microbiota restores hematopoiesis in antibiotic-treated mice.[16] Furthermore, we have shown that the bone marrow of STAT1-deficient mice phenocopies that of antibiotic-treated mice, indicating that the microbiota supports normal hematopoiesis via STAT1 signaling. These results are consistent with earlier studies linking STAT1 to regulation of hematopoiesis.[23]

Despite this prior work, the mechanisms by which the microbiota contributes to normal hematopoiesis remain poorly understood. Specifically, by what mechanism does the microbiota signal to HSPCs in the distant bone marrow? Which cellular components sense these signals, and how are they translated to affect hematopoietic activity? The current study focuses on defining the molecular mechanisms and signaling pathways by which the microbiota promotes normal hematopoiesis, a necessary step to inform preventive and therapeutic approaches for antibiotic-associated bone marrow suppression. We sought to better understand both the specific microbial metabolites and cytokine signaling contributing to microbiota-dependent maintenance of hematopoiesis. Conditional knockout and oral supplementation studies were used to define pathways involved in antibiotic-mediated bone marrow suppression. These studies indicate that the microbiota supports hematopoiesis via type I interferon (IFN)-induced STAT1 signaling in hematopoietic progenitors and reveal microbiota-derived products that may rescue antibiotic-induced cytopenias.

Methods

Mice

We used 6- to 10-week-old CD45.1$^+$ or CD45.2$^+$ C57BL/6 mice for all experiments. Mice were bred and housed in SPF animal facilities at Baylor College of Medicine (BCM) and Washington University in accordance with protocols approved by the respective Institutional Animal Care and Use Committees at BCM (AN-4802) and Washington University (20190162). At BCM, all mice have free access to standard chow, 5V5R (Lab Supply). At Washington University, all mice have free access to standard chow, PicoLab Rodent Diet 5053 (Lab Supply). *Stat1*$^{-/-}$ mice [B6.129S(Cg)-Stat1-tm1Div/J stock #012606 obtained from The Jackson Laboratory], *Ifnar1*$^{-/-}$ mice,[24] *Ifngr1*$^{-/-}$ mice,[25] and *Ifnlr1*$^{-/-}$ mice[26] were bred, maintained, and treated at BCM or Washington University. *Stat1*$^{fl/fl}$ [B6;129S-*Stat1*^{tm1Mam}/Mmjax] or *Ifnar1*$^{fl/fl}$ [B6(Cg)-Ifnar1tm1.1Ees/J] mice were crossed to Vav-iCre mice [B6.Cg-Commd10Tg(Vav1-icre)A2Kio/J], LysM-Cre mice,[27] Villin-Cre mice,[28] and LepR-Cre mice [B6.129(Cg)-Leprtm2(cre)Rck stock #008320, obtained from The Jackson Laboratory] and Cre-positive and Cre-negative littermates were used in experiments.

Antibiotic treatment

Mice were housed in mixed bedding combined from all cages for 2 weeks before the start of antibiotic treatment or, for experiments with conditional knockout animals, Cre-negative and Cre-positive littermates were cohoused before the start of the antibiotics.

At the start of antibiotic treatment, drinking water was replaced with water containing antibiotics: 0.5 g/L vancomycin, 1 g/L neomycin, 1g/L ampicillin, and 1 g/L metronidazole (VNAM; Sigma Aldrich). Flavoring (20 g/L grape-flavored Kool-Aid Drink Mix; Kraft Foods Global, Inc.) was added to the drinking water for all groups. Treatment continued for 14 days, with VNAM or Kool-Aid provided ad libitum for the duration.

Flow cytometry for blood and bone marrow populations

Bone marrow was obtained by flushing leg bones. Bone marrow cells were stained (details are provided in the supplemental Methods) and analyzed on an LSRII flow cytometer (BD Biosciences).

NOD1/2 ligand treatment

Control and antibiotic-treated mice were treated with 100 μg of C12-iE-DAP ("NOD1L") (InvivoGen) in 200 μL of water or 300 μg of muramyl dipeptide ("NOD2 agonist") (MDP; InvivoGen) in 150 μL of water by oral gavage every 2 to 3 days on days 7 to 14 of antibiotic treatment before analysis on day 14.

Cell culture

32D Clone 3 cells (ATCC) were grown in RPMI 1640 medium with 2 mM GlutaMax (Gibco-ThermoFisher) supplemented with 1% *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 1% sodium pyruvate, 10% fetal bovine serum (Gibco-ThermoFisher), 1% penicillin/streptomycin (Invitrogen), and 10 ng/mL of mouse interleukin-3 (PeproTech). Then, 1 × 10^6 32D cells were cultured with L-proline

(150 μM; Sigma-Aldrich), L-α-phosphatidylcholine (100 μM; Sigma-Aldrich), orotic acid (10 μM; Sigma-Aldrich), or γ-glutamyl-L-alanine (100 μM, MedChemExpress) for 4 hours in interleukin-3–free complete RPMI medium, with glycerol (100 μM; EMD Chemicals Inc.) as the negative control and recombinant murine IFN-α2 (10 ng/mL; eBioscience) as the positive control.

Quantitative polymerase chain reaction assays

Approximately 1.5×10^6 murine cKit$^+$ (CD117) cells were isolated by enrichment using anti-CD117 magnetic microbeads (Macs, Miltenyi) from bone marrow of mice treated with VNAM and NOD1 ligand (NOD1L) or control and were suspended in TRIzol (Invitrogen). RNA was isolated with isopropanol and ethanol. Complementary DNA was generated with random hexamers using SuperScript IV Reverse Transcriptase (Invitrogen). Expression of *Ifitm3* or *Stat1* was quantified by using SYBR Green Master Mix (Bio-Rad) as measured on the Roche LightCycler 96 (Roche). The supplemental Methods provide additional details.

Transplantation assays

Whole bone marrow was obtained from CD45.2$^+$ *Stat1*$^{-/-}$ or wild type (WT) mice by flushing leg bones from a single donor with ∼5 mL per bone of Hanks buffered saline solution (Gibco-Life Technologies) with 1% penicillin/streptomycin (Invitrogen) and 1% *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid buffer (Gibco-ThermoFisher). Recipient CD45.1$^+$ WT or CD45.2$^+$ *Stat1*$^{-/-}$ mice were irradiated with 10.5 Gy split into 2 doses 4 hours apart. Then, 2×10^6 cells from each donor were injected into recipients via a retro-orbital intravenous injection. Engraftment was assessed at 8 weeks posttransplant by using retro-orbital bleed and flow cytometry for lineage composition as previously described.[16] At 10 weeks, mice housed in mixed bedding were split into control or antibiotic-treated groups. Antibiotic treatment (as noted earlier) began at 12 weeks.

Untargeted metabolomics

Fecal pellets and serum were collected from individual mice immediately before and 2 weeks after stopping treatment with VNAM. Metabolites were extracted from stool samples, and mass spectrometry was performed (details are provided in the supplemental Methods).

Statistical analysis

Results are reported as mean ± SEM. Results with 2 groups were analyzed with the Mann-Whitney *U* test for nonparametric data and Welch's *t* test for nonparametric data with unequal variances. Statistical analysis for 3 groups or more were analyzed by using one-way analysis of variance with Tukey's correction for multiple comparisons for parametric data and Kruskal-Wallis test with Dunn's multiple comparison test for nonparametric data. For untreated and antibiotic-treated groups and means for the different genotypes, results were analyzed by using a two-way analysis of variance with Šidák's multiple comparison test comparing means. Results with *P* values $<.05$ were considered statistically significant.

Full methods are available in the supplemental Methods.

Results

STAT1 signaling is required in the hematopoietic compartment for the microbiota to promote normal hematopoiesis

Our prior work showed that the microbiota induces STAT1 signaling to support normal hematopoiesis but did not reveal the cellular compartment in which STAT1 signaling is relevant.[16] The host cells in closest proximity to the intestinal microbiota are intestinal epithelial cells. To test if STAT1 signaling in intestinal epithelial cells affects antibiotic-induced suppression of hematopoiesis, we treated *Villin-Cre;Stat1*$^{fl/fl}$ mice or Cre-negative controls with an oral antibiotic cocktail consisting of vancomycin, neomycin, ampicillin, and metronidazole (VNAM) or mock treatment for 2 weeks (supplemental Figure 1A). Confirming findings from our prior study,[16] multipotent progenitor populations of Cre-negative mice were suppressed upon VNAM treatment compared with those that received mock treatment, reflecting hematopoietic suppression (supplemental Figure 1B). Because multipotent progenitor populations comprise the majority of the Lin$^-$Sca$^+$cKit$^+$ (LSK) population of the bone marrow, we subsequently observed the LSK population as an indicator of normal hematopoiesis in our model. The LSK population was normal in *Villin-Cre*;*Stat1*$^{fl/fl}$ mice, and knocking out *Stat1* in intestinal epithelial cells did not affect VNAM-mediated suppression of LSK numbers (supplemental Figure 1C). These data indicate that STAT1 activation in nearby intestinal epithelial cells is not required to promote normal microbially supported hematopoiesis.

A study implicated mesenchymal stromal cells (MSCs) in the transduction of microbial signals to support hematopoiesis.[29] To test if STAT1 signaling in MSCs affects antibiotic-induced suppression of hematopoiesis, we assessed the LSK compartment of *LepR-Cre*;*Stat1*$^{fl/fl}$ mice in the presence or absence of VNAM treatment. The LSK compartment in *LepR-Cre*;*Stat1*$^{fl/fl}$ mice was similar to that of Cre-negative and WT controls, and LSK suppression occurred similarly to WT in VNAM-treated *LepR-Cre*;*Stat1*$^{fl/fl}$ mice, suggesting that STAT1 signaling in MSCs is not necessary for normal hematopoiesis (supplemental Figure 1D).

In light of these negative data, we evaluated LSK cells and the response to antibiotics in *Vav-iCre;Stat1*$^{fl/fl}$ mice, in which *Stat1* is disrupted in hematopoietic and endothelial cells.[30] We found that LSK populations of Cre-positive mice were decreased compared with Cre-negative mice, and the percentage and absolute numbers of LSK cells in Cre-positive mice were not further suppressed upon receipt of 2 weeks of VNAM compared with mock treatment (Figure 1A; supplemental Figure 1E). Altogether, these data suggest that the intestinal microbiota stimulates hematopoiesis by activating STAT1 signaling in the hematopoietic compartment, thus bypassing both intestinal epithelial cells and MSCs in this process.

To confirm the role of STAT1 specifically in the hematopoietic compartment, we performed bone marrow transplants in which CD45.2$^+$ *Stat1*$^{-/-}$ or CD45.2$^+$ WT whole bone marrow was noncompetitively transplanted into lethally irradiated CD45.1$^+$ WT recipient mice (Figure 1B). After 12 weeks of reconstitution, transplanted mice were treated with VNAM or mock treatment for 2 weeks. As expected, control mice transplanted with WT bone marrow had normal levels of LSK populations, and the LSK compartment was suppressed when these mice received VNAM treatment.[16] In contrast, the LSK compartment of mice receiving

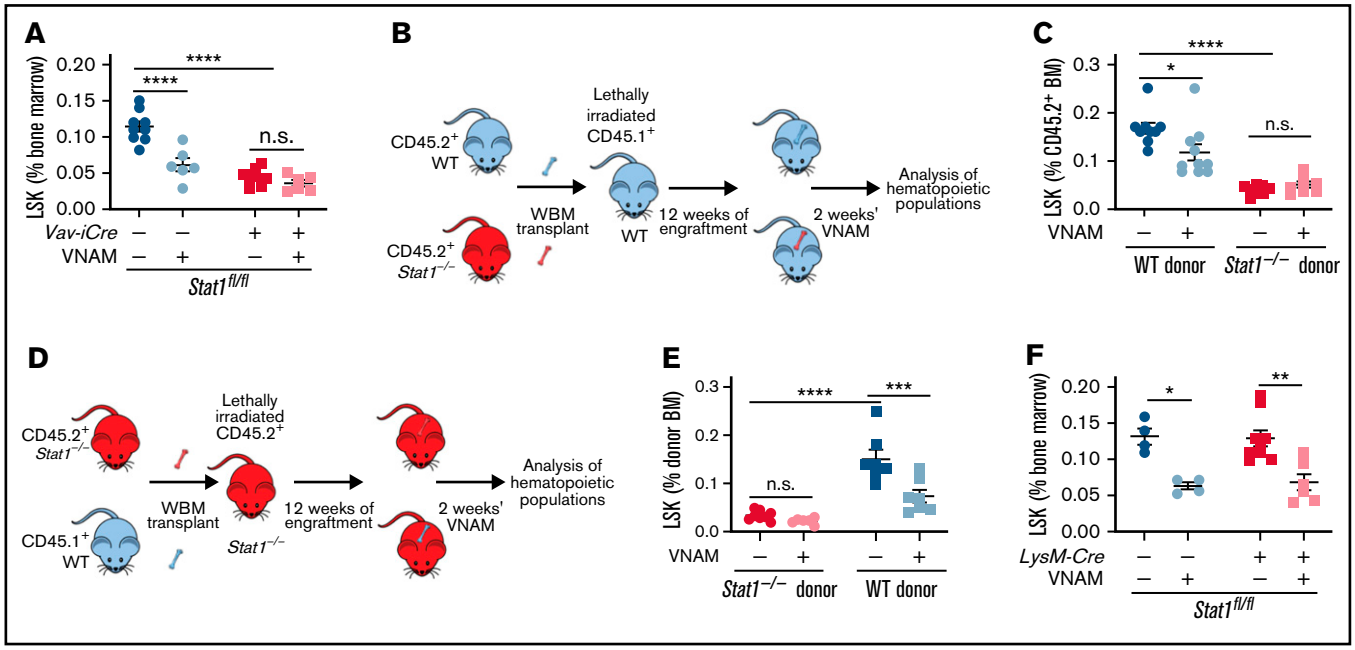

Figure 1. STAT1 signaling is required in non-myeloid hematopoietic cells for microbiota-mediated hematopoiesis. (A) LSK populations were quantified from mice with or without STAT1 signaling in *Vav-iCre*;*Stat1*$^{fl/fl}$ mice after 2 weeks with or without VNAM according to flow cytometry. (B) Then, 2 × 10^6 whole bone marrow (WBM) cells from CD45.2$^+$ *Stat1*$^{-/-}$ or WBM cells from WT mice were injected retro-orbitally into lethally irradiated CD45.1$^+$ WT recipient mice. Twelve weeks after engraftment, the chimeric mice received 2 weeks of either VNAM treatment or mock treatment. (C) LSK populations were quantified from CD45.1$^+$ recipient mice that received either WT or *Stat1*$^{-/-}$ bone marrow (BM) and were treated with 2 weeks of VNAM after 12 weeks of engraftment according to flow cytometry. (D) Then, 2 × 10^6 WBM cells from either CD45.1$^+$ WT or CD45.2$^+$ *Stat1*$^{-/-}$ mice were injected retro-orbitally into lethally irradiated CD45.2$^+$ *Stat1*$^{-/-}$ recipient mice. Twelve weeks after engraftment, the chimeric mice received 2 weeks of either VNAM treatment or mock treatment. (E) BM progenitor populations were quantified from CD45.2$^+$ recipient mice that received either CD45.1$^+$ WT or CD45.2$^+$ *Stat1*$^{-/-}$ BM and were treated with 2 weeks of VNAM after 12 weeks of engraftment according to flow cytometry. (F) LSK populations were quantified from mice with or without STAT1 signaling in *LysM-Cre*;*Stat1*$^{fl/fl}$ mice after 2 weeks with or without VNAM according to flow cytometry. Results are compiled from 2 or 4 independent experiments (n = 4-10 per group) (panels A and F) or representative of 2 independent experiments (n = 7-10 [panel C] or n = 6-8 per group [panel E]). Graphs show mean ± SEM, with statistical significance determined by two-way analysis of variance with Šidák's multiple comparisons test. *P < .05, **P < .01, ***P < .001, ****P < .0001. n.s., not significant.

Stat1$^{-/-}$ donor marrow was suppressed at baseline, and VNAM treatment did not induce further suppression (Figure 1C), phenocopying prior findings in germline *Stat1*$^{-/-}$ mice. This result indicates that the microbiota induces STAT1 signaling specifically in the hematopoietic compartment to promote normal hematopoiesis.

To confirm that STAT1 signaling is not required in the non-hematopoietic system during steady-state hematopoiesis, we also transplanted CD45.1$^+$ WT or CD45.2$^+$ *Stat1*$^{-/-}$ whole bone marrow into lethally irradiated CD45.2$^+$ *Stat1*$^{-/-}$ recipient mice (Figure 1D). Twelve weeks after transplantation, transplant recipients were treated with VNAM or mock treatment. Whereas *Stat1*$^{-/-}$ mice receiving *Stat1*$^{-/-}$ marrow had suppressed LSK populations at baseline or after VNAM, mice that received WT donor marrow had normal populations of LSK that were suppressed upon VNAM treatment (Figure 1E). These findings confirm that microbiota-induced STAT1 signaling is not required in radioresistant tissues but occurs specifically in the hematopoietic compartment. Collectively, our transplant and conditional knockout studies confirm that STAT1 signaling is necessary only in the hematopoietic compartment for the microbiota to promote steady-state hematopoiesis.

As myeloid cells have been shown to participate actively in the regulation of HSPCs, including acting as an intermediate in microbially promoted hematopoiesis, we assessed their role in this phenomenon.[31-34] Interestingly, *LysM-Cre;Stat1*$^{fl/fl}$ mice,[35] which lack myeloid STAT1, exhibited normal LSK numbers that were reduced upon VNAM treatment, similar to Cre-negative controls (Figure 1F). These findings suggest that the hematopoietic cells in which STAT1 normally supports primitive hematopoiesis are non-myeloid.

Type I IFN signaling is uniquely required for the microbiota to support normal hematopoiesis

The transcription factor STAT1 mediates downstream signaling for all IFNs, including type I, II, and III IFNs.[36,37] To define the specific IFN signaling pathway(s) that rely upon STAT1 signaling to regulate hematopoiesis, we treated mice deficient in individual IFN receptors (*Ifnar1*$^{-/-}$, *Ifngr1*$^{-/-}$, and *Ifnlr1*$^{-/-}$) with VNAM or mock treatment. Of the 3 mouse lines, *Ifngr1*$^{-/-}$ and *Ifnlr1*$^{-/-}$ appeared similar to WT mice, with normal starting percentages of LSK cells and significant suppression of the LSK population upon VNAM treatment (Figure 2A-B). In contrast, *Ifnar1*$^{-/-}$ mice, which lack active type I IFN signaling, phenocopied *Stat1*$^{-/-}$ mice with equally low LSK numbers after mock or VNAM treatment. These results in *Ifnar1*$^{-/-}$ mice suggest that STAT1 functions downstream of type I IFN signaling in the hematopoietic response to the microbiota.

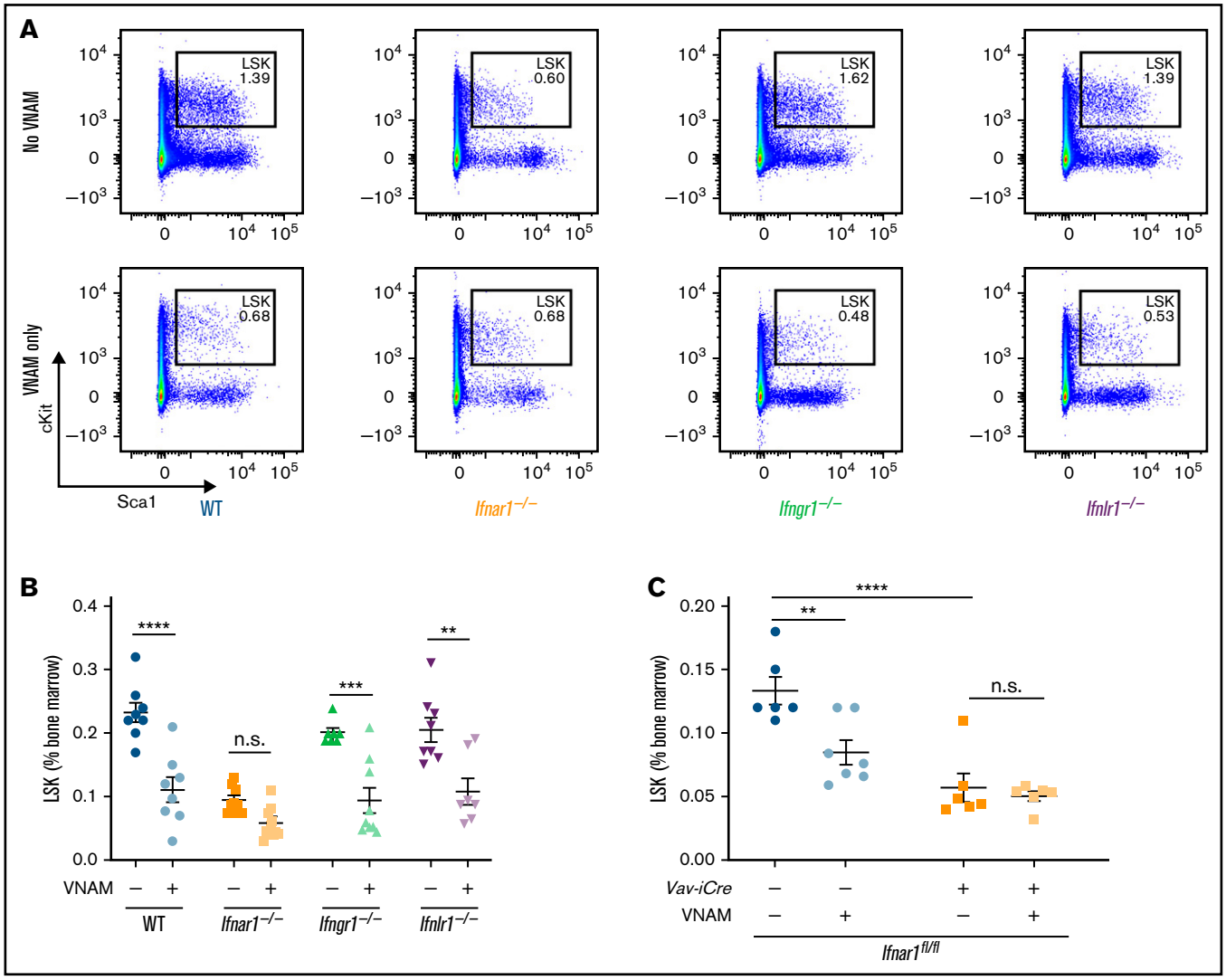

Figure 2. Type I (not type II or III) IFN signaling is required for microbiome-mediated hematopoiesis. (A) LSK populations from mice with or without type I (*Ifnar1*$^{-/-}$), type II (*Ifngr1*$^{-/-}$), or type III (*Ifnlr1*$^{-/-}$) IFN signaling after 2 weeks with or without VNAM (B) were quantified. (C) LSK populations were quantified from mice with or without type I IFN signaling in *Vav-Cre;Ifnar1*$^{fl/fl}$ mice after 2 weeks with or without VNAM according to flow cytometry. Results are compiled from 2 independent experiments (n = 7-11 per group; panels A and B) or from 3 independent experiments (n = 6-7; panel C). Graphs show mean ± SEM, with statistical significance determined by two-way analysis of variance with Šidák's multiple comparisons test. **$P < .01$, ***$P < .001$, ****$P < .0001$. n.s., not significant.

To assess whether type I IFN signaling is responsible for the promotion of hematopoiesis by STAT1 in hematopoietic cells, we treated *Vav-iCre;Ifnar1*$^{fl/fl}$ mice with VNAM or mock treatment. Similar to the *Vav-iCre;Stat1*$^{fl/fl}$ mice, LSK populations from Cre-positive mice were suppressed compared with Cre-negative controls, and the LSK compartment was not further suppressed upon VNAM treatment (Figure 2C). These data indicate that type I IFN signaling is specifically required in the hematopoietic compartment and are consistent with a model in which the microbiota mediates normal hematopoiesis by activating type I IFNs followed by STAT1 signaling in hematopoietic progenitor cells.

We next sought to rescue the hematopoietic defects caused by antibiotic treatment by stimulating the type I IFN pathway. Steed et al[38] previously reported that the microbiota-derived molecule desaminotyrosine (DAT), made by the commensal gut bacterium *Clostridium orbiscindens,* increases the survival of antibiotic-treated mice during influenza infection in a type I IFN–dependent manner. In addition, DAT is less abundant in antibiotic-treated mice.[39] We therefore fed mice DAT (20 mmol) via oral gavage on days 7, 9, and 12 after the start of VNAM treatment (supplemental Figure 2A). Bone marrow granulocytes were significantly higher in mice given DAT with VNAM compared with VNAM only (supplemental Figure 2B), but there was no increase in bone marrow progenitors from mice receiving DAT (supplemental Figure 2C). These findings suggest that oral supplementation of DAT, an inducer of type I IFN, is sufficient to rescue the granulocytic defects but not the hematopoietic progenitor suppression seen in antibiotic-treated mice.

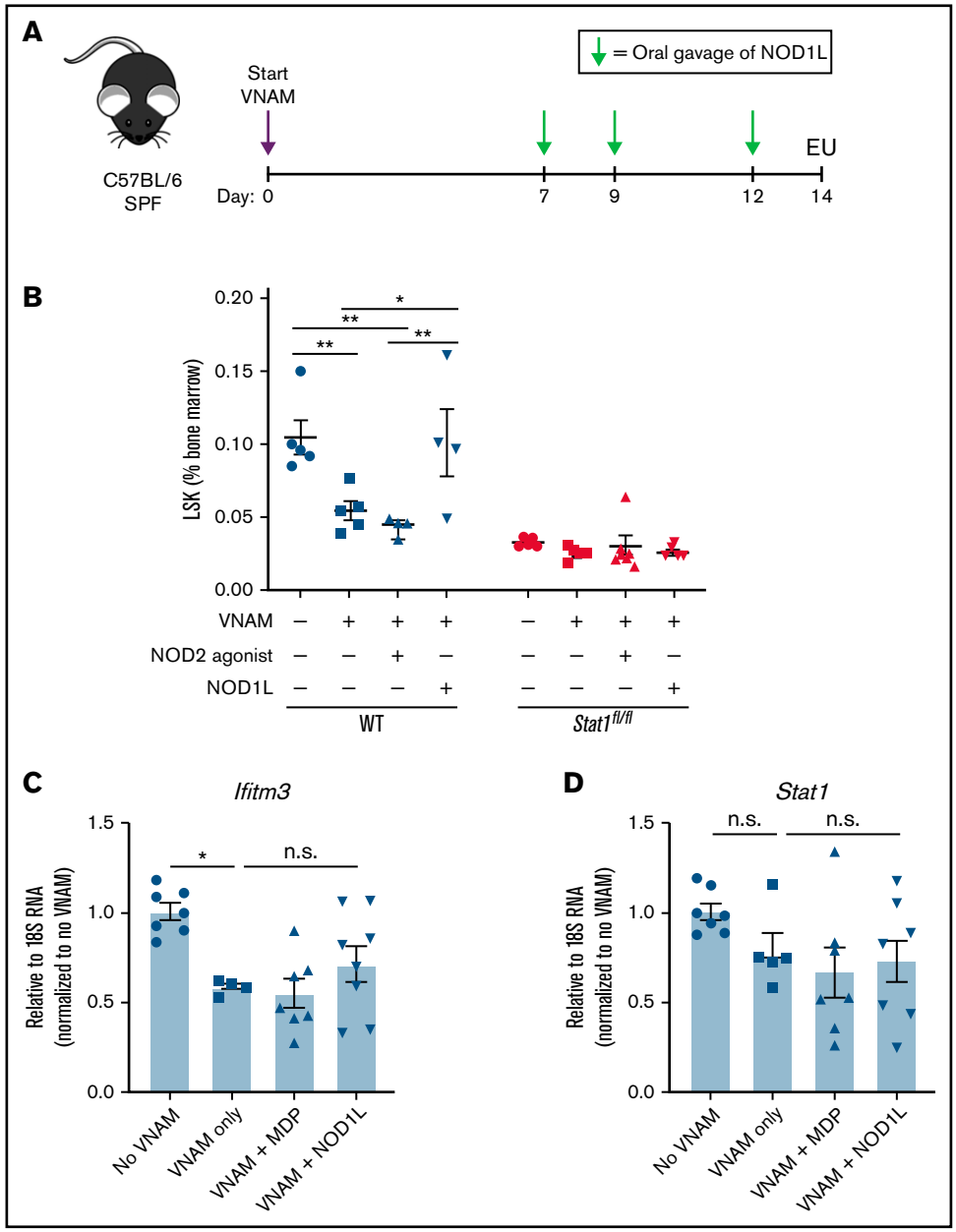

Figure 3. Exogenous NOD1L treatment can rescue hematopoietic progenitor defects of mice on antibiotic therapy. (A) Treatment strategy of NOD1L treatment with mice euthanized (EU) at the end of VNAM treatment. (B) Bone marrow progenitors were measured from WT or *Stat1*$^{-/-}$ mice that received either 2 weeks of mock treatment or VNAM as well as NOD1L (100 μg) or NOD2 agonist (300 μg) as control on the second week every 2 to 3 days. *Ifitm3* (C) and *Stat1* (D) gene expression of cKit^{+} cells harvested using Auto-MACS from mice that received either 2 weeks of mock treatment or VNAM as well as NOD1L (100 μg) or NOD2 agonist (300 μg) as control on the second week every 2 to 3 days as quantified with quantitative polymerase chain reaction. Results are representative of 3 independent experiments (n = 4-6 per group; panel B) or compiled from 2 independent experiments (n = 4-8 mice per group; panels C and D). Graphs show mean ± SEM, with statistical significance determined by two-way analysis of variance with Šidák's multiple comparisons test (panel B) or one-way analysis of variance with Tukey's multiple comparison test (panels C and D). *$P < .05$, **$P < .01$, n.s., not significant.

Oral supplementation of NOD1L rescues bone marrow progenitor and granulocytic defects in antibiotic-treated mice

Another microbially derived product that induces type I IFN is NOD1L.[40,41] Interestingly, Iwamura et al[29] showed that stimulation of the pattern recognition receptor NOD1 by NOD1L, which is derived from peptidoglycan,[42] rescues the hematopoietic defects of germ-free mice. We thus hypothesized that NOD1L could rescue the hematopoietic defects in VNAM-treated mice. We fed WT or *Stat1*$^{-/-}$ mice NOD1L, C12-iE-DAP (100 μg), via oral gavage on days 7, 9, and 12 after initiation of VNAM treatment (Figure 3A).

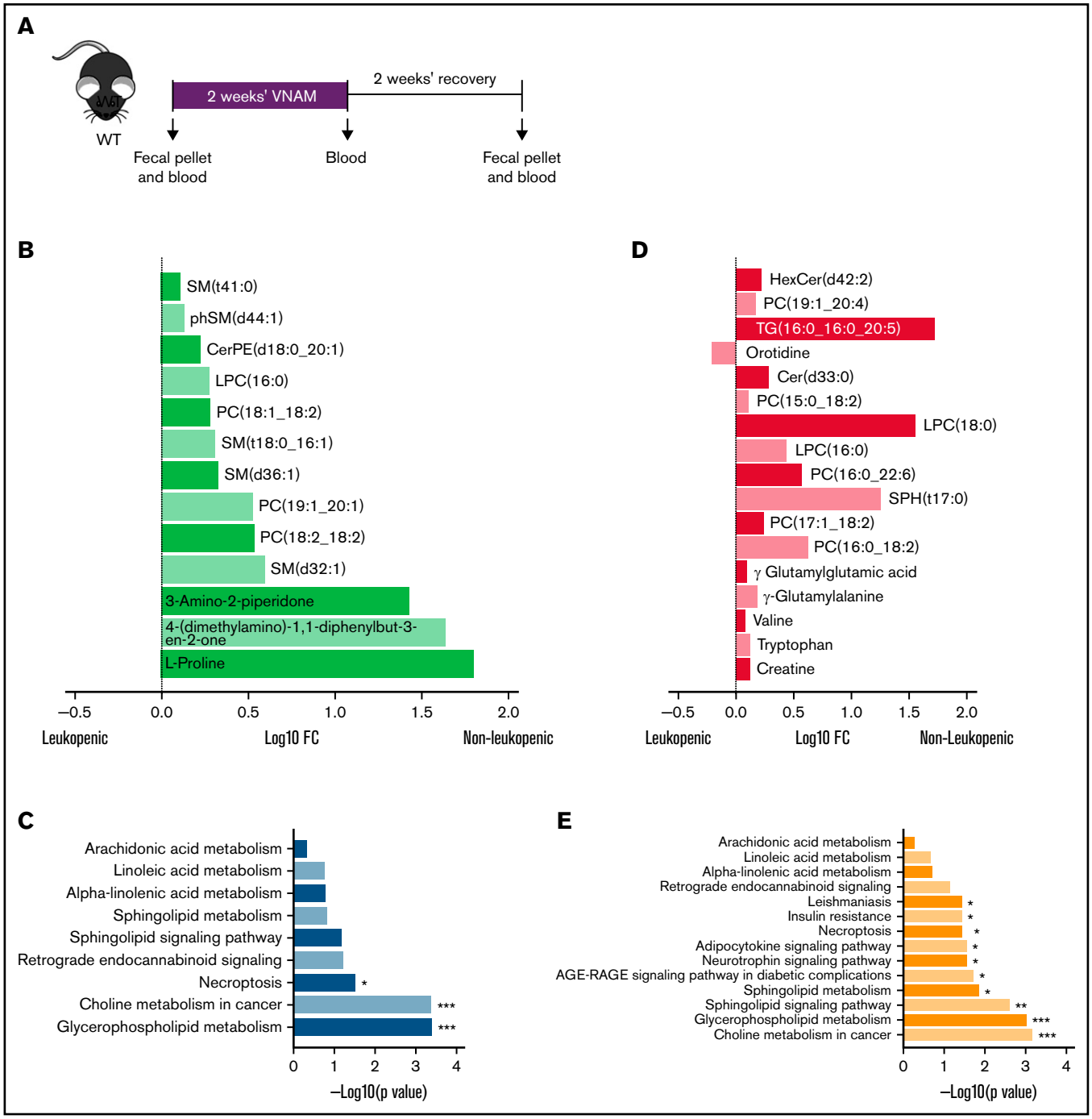

Figure 4. Metabolomics comparison of leukopenic and non-leukopenic mice reveals additional microbial products that may support hematopoiesis. (A) Experimental schematic of stool and serum collection for metabolomics. (B) Metabolites of interest generated from nontargeted metabolomics of stool. (C) The top enriched LIPEA pathway results from the fecal metabolomics. (D) Metabolites of interest generated from nontargeted metabolomics of serum. (E) The top enriched LIPEA pathway results from the serum metabolomics. FC, fold change. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$.

The NOD2 agonist MDP (300 μg) was used as a negative control, as *Nod2* has previously been shown to be dispensable for microbiota-promoted hematopoiesis.[16] The LSK population was suppressed in WT mice that received VNAM alone or VNAM with the NOD2 agonist but was restored in the presence of NOD1L supplementation (Figure 3B). The LSK populations from *Stat1*$^{-/-}$ mice were suppressed at baseline, and neither VNAM treatment nor the addition of MDP or NOD1L restored LSK cells, indicating that NOD1L acts in a STAT1-dependent manner to support hematopoietic progenitor populations.

We further examined granulocyte populations in VNAM- and NOD1L-treated mice. Bone marrow granulocytes were suppressed

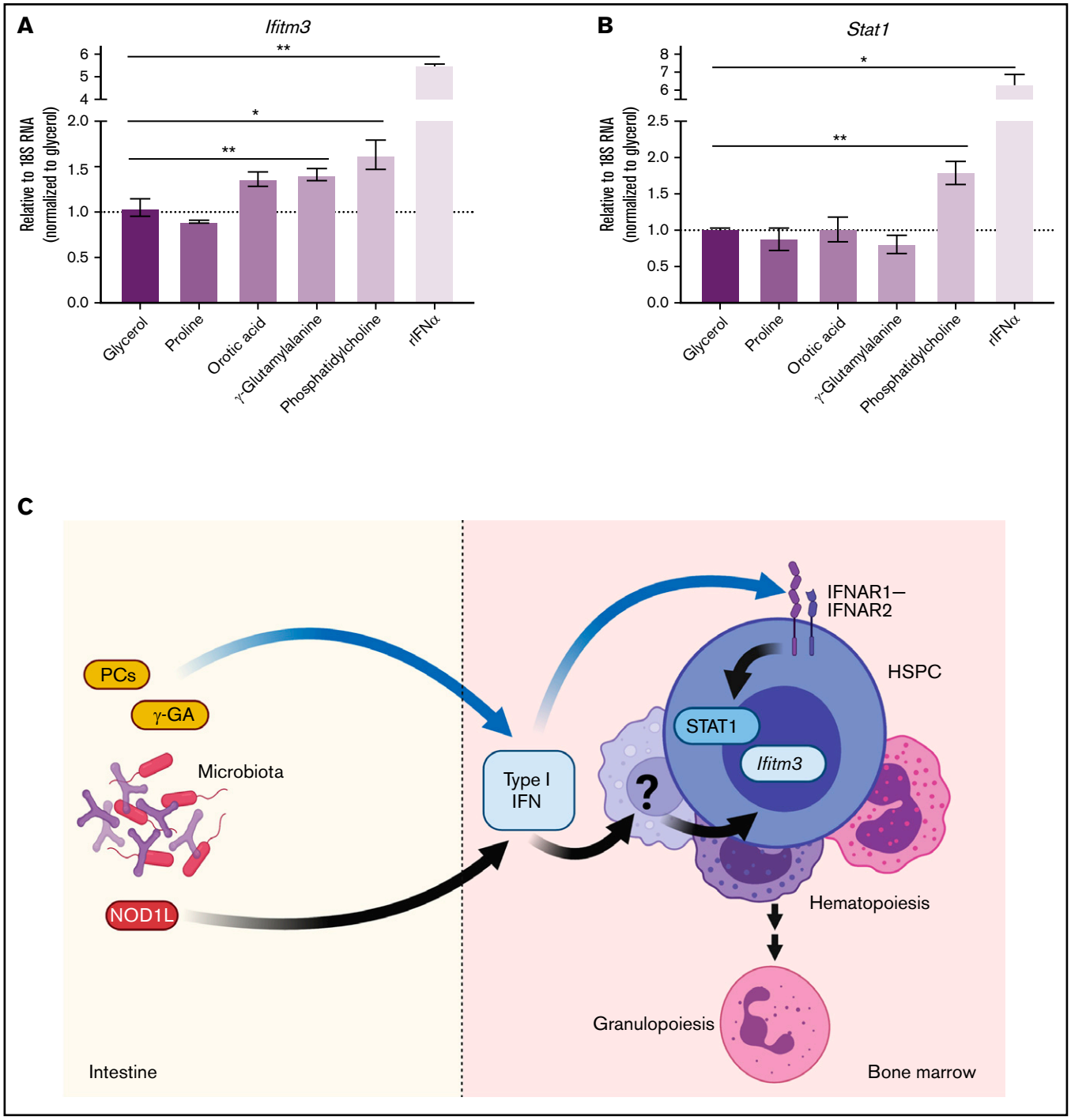

Figure 5. Phosphatidylcholines (PCs) and γ-glutamylalanine can induce expression of type I IFN response genes. Expression of IFN-stimulated genes *Ifitm3* (A) and *Stat1* (B) were quantified in 32D cells stimulated with compounds of interest from the metabolomics screens proline (150 μM), PC (100 μM), orotic acid (10 μM), or γ-glutamylalanine (300 μM) for 4 hours with glycerol (0.1 mM) as the negative control and recombinant murine IFN-α2 (10 ng/mL) as the positive control. (C) Proposed model of microbiota-dependent hematopoiesis created with Biorender.com. Results are compiled from individual independent experiments normalized to their respective glycerol control for statistics (n = 3-12 technical replicates per group). Graphs show mean ± SEM, with statistical significance determined by one-way analysis of variance with Dunnett's multiple comparison test, Kruskal-Wallis test with Dunn's multiple comparison test (orotic acid and PC for *Ifitm3*), or Welch's *t* test (recombinant IFN-α [rIFN-α]). **P* < .05, ***P* < .01. γ-Ga, γ-glutamylalanine.

in WT mice in the presence of VNAM as expected[16] but rescued by the administration of NOD1L (supplemental Figure 3A). Notably, this rescue also occurred in *Stat1*$^{-/-}$ mice, which exhibited a restoration of granulocyte numbers upon NOD1L treatment but not NOD2 agonist treatment. To confirm that growth of antibiotic-resistant bacteria was not responsible for the restoration of

granulocyte numbers, we performed quantitative polymerase chain reaction to detect copies of the 16S rRNA gene in the stool and found that the microbiota remained suppressed in VNAM + NOD1L–treated mice, similar to those treated with VNAM alone (supplemental Figure 3B). These findings suggest that NOD1L-mediated granulopoiesis can occur via a STAT1-independent mechanism, independent of its effects on the LSK progenitor compartment. We also noted that NOD1L alone did not increase granulocyte numbers compared with control WT mice (supplemental Figures 3C-D), indicating that exogenous NOD1L can rescue granulocyte depletion in the context of microbiota disruption but does not cause granulocyte expansion when the microbiota is intact. Collectively, these findings indicate that the microbial product NOD1L supports both early hematopoietic progenitors and downstream granulopoiesis.

To assess whether basal type I IFN signaling in hematopoietic progenitors is affected by antibiotic treatment and/or NOD1L, we collected cKit$^+$ cells from mice treated with VNAM and NOD1L, isolated RNA, and generated complementary DNA for quantitative real-time polymerase chain reaction. We assessed expression of *Ifitm3* and *Stat1*, which are 2 of the most highly expressed type I IFN–stimulated genes in hematopoietic progenitor cells. Expression of *Ifitm3* was shown to be significantly decreased in the hematopoietic progenitor cells of VNAM-treated mice compared with cells from mock-treated mice, and the expression of *Stat1* trended lower (Figure 3C-D). These data indicate that basal type I IFN signaling is diminished in hematopoietic progenitor cells upon VNAM treatment, and they serve as the first evidence that the microbiota can induce type I IFN signaling to support hematopoiesis in the bone marrow. Surprisingly, expression of *Ifitm3* and *Stat1* was not significantly different in the hematopoietic progenitor cells of mice treated with VNAM and NOD1L compared with those treated with VNAM alone. Although these results may be hampered by high biological variability between the samples, they are insufficient to conclude that NOD1L triggers type I IFN signaling in hematopoietic progenitors to promote hematopoiesis. These data suggest either that a cKit$^-$ population of hematopoietic cells transmits NOD1L-dependent type I IFN signaling.

Metabolomics comparison of leukopenic and non-leukopenic mice reveals additional microbial products that may support hematopoiesis

Although we showed that NOD1L is sufficient to rescue hematopoietic defects induced by VNAM treatment, we did not find that NOD1L treatment restored type I IFN signaling in HSPCs, and we hypothesized that other products made by the microbiota could also contribute to steady-state hematopoiesis. We therefore performed untargeted metabolomics on murine stool and serum samples to identify additional candidates. Mice were treated with VNAM for 2 weeks to suppress bone marrow and then allowed to recover for 2 weeks (Figure 4A). As previously observed,[16] the peripheral leukocyte counts of VNAM-treated mice recovered heterogeneously, with about one-half of the cohort recovering to pretreatment peripheral counts 2 weeks after antibiotic cessation (supplemental Figure 4A). Fully recovered mice were considered non-leukopenic, whereas those that did not reach pretreatment counts were considered leukopenic. We then performed metabolomics analysis of stool and serum comparing the leukopenic and non-leukopenic groups, reasoning that the leukopenic group may be missing a metabolite important for hematopoiesis. Analysis of stool metabolites revealed 13 metabolites present in the non-leukopenic group but depleted from the leukopenic mice (P exact $< .05$) (Figure 4B; supplemental Figure 4B). Among these metabolites, we identified proline, which was the most abundant metabolite in the non-leukopenic group, and several phosphatidylcholines (18:1_18:2, 19:1_20:1, and 18:2_18:2), as well as largely unstudied metabolites such as 3-amino-2-piperidone and 4-(dimethylamino)-1,1-diphenylbut-3-en-2-one. Hierarchical clustering did not reveal broad similarities or metabolic pathways differentiating samples from leukopenic vs non-leukopenic mice (supplemental Figure 4C). The lipids differentially identified among stool metabolites were associated with necroptosis, choline metabolism in cancer, and glycerophospholipid metabolism pathways, according to Lipid Pathway Enrichment Analysis (LIPEA) (Figure 4C). These pathways have not been previously reported to influence steady-state hematopoiesis.

The analysis of serum metabolites revealed 16 metabolites present in the serum of the non-leukopenic group but depleted in leukopenic mice (P-adjusted $< .05$) (Figure 4D; supplemental Figure 4D). Similar to the stool metabolomics data, hierarchical clustering of serum metabolomics data did not reveal broad similarities differentiating samples from leukopenic vs non-leukopenic mice (supplemental Figure 4E). Among the differentially detected metabolites, phosphatidylcholines accounted for nearly one-half of the lipids. Of the nonlipid metabolites, γ-glutamylalanine was the most abundant in the non-leukopenic group relative to the leukopenic group (Figure 4D). Similar to the stool metabolomics analysis, LIPEA pathway analysis revealed that serum lipids identified at higher levels in non-leukopenic vs leukopenic mice were involved in glycerophospholipid metabolism (Figure 4E). Interestingly, orotic acid, the precursor of orotidine (P-adjusted $= .0268$), was higher in the leukopenic mice compared with the non-leukopenic mice (P-adjusted $= .0524$) (supplemental Figure 4D), suggesting that this compound could be suppressive.

Phosphatidylcholines and γ-glutamylalanine can induce expression of type I IFN response genes *Ifitm3 and Stat1*

To ascertain whether any of the metabolites identified in our metabolomics screens might induce type I IFN and STAT1 signaling in hematopoietic cells, we cultured 32D cells with biologically relevant concentrations of several compounds. Expression of *Ifitm3* and *Stat1* in response to stimulation with proline or orotic acid was not statistically different from that of the glycerol negative controls (Figure 5A-B). In contrast, phosphatidylcholine exposure induced expression of *Ifitm3* and *Stat1*, and treatment with γ-glutamylalanine induced expression of *Ifitm3*. These results suggest that phosphatidylcholine and γ-glutamylalanine can stimulate the expression of the type I IFN pathway response genes in hematopoietic cells and are microbial compounds that could potentially contribute to maintenance of hematopoiesis.

Discussion

The current study identified key interactions through which the microbiota promotes steady-state hematopoiesis. Using transgenic and bone marrow chimeric mice, we determined that type I IFN–STAT1 signaling in hematopoietic cells, not the intestinal epithelium or MSCs, is critical for the intestinal microbiota to promote

hematopoiesis. Indeed, we found that basal type I IFN signaling is diminished in hematopoietic progenitors with antibiotic treatment and that the microbial product NOD1L can restore hematopoietic progenitor and bone marrow granulocyte populations in the setting of ongoing antibiotic treatment. Crucially, this work shows that administration of microbial products such as NOD1L can prevent the development of cytopenias, presenting an attractive and novel therapeutic strategy for patients. Finally, we identified metabolites in the nontargeted metabolomics screen that have not been previously described to be associated with primitive hematopoiesis, 2 of which can induce type I IFN signaling, increasing the range of candidates available to restore hematopoiesis in patients receiving prolonged antibiotics.

We identified type I IFNs as the key IFN mediating microbiota-driven hematopoiesis. Type I IFNs previously have been shown to activate dormant hematopoietic stem cells at steady state, and chronic stimulation of this pathway may lead to stem cell exhaustion.[43-45] Type I IFNs are also detrimental in the context of infection with pathogens such as *Ixodes ovatus Ehrlichia*, leading to hematopoietic collapse via induction of stem cell death and quiescence.[46] However, our data indicate that basal type I IFN signaling is crucial to maintain hematopoietic progenitor populations in the context of microbiota-promoted steady-state hematopoiesis. The dosage and extent of type I IFN stimulation likely explain this difference, as IFNs are activated only to low levels by the microbiota at steady state. Low-level IFN activation primes the immune system to respond to future infection without the potentially damaging effects of large-scale IFN induction following infection.[47,48]

Our results indicate that IFN signaling within the hematopoietic compartment is key for steady-state hematopoiesis (Figures 1 and 2), and our data support that this action is due to direct effects on hematopoietic progenitors (Figure 3), as suggested by previous studies.[43] However, it is possible that non-myeloid mature blood cells such as innate lymphoid type 2 cells may also contribute to regulation of the hematopoietic compartment in a STAT1-dependent manner, and further studies are necessary to define the contributions of these cells.[49]

We found that stimulating NOD1, but not NOD2, promotes hematopoiesis in the absence of the microbiota in a STAT1-dependent manner. These data clarify previous reports that identified multiple innate immune pathways, including NOD1 and STAT1, as separately promoting hematopoiesis in a microbiota-dependent manner.[16,29] Although NOD1 and NOD2 possess distinct agonists, they have similar regulons and effects, and both are capable of activating type I IFN signaling.[41,50] Interestingly, NOD1Ls, which contain iE-DAP, are primarily found within peptidoglycan of gram-negative bacteria, whereas NOD2 ligands, which contain MDP, are found in both gram-negative and gram-positive bacteria.[51] This distinction may indicate that gram-negative commensals have a more central role in steady-state hematopoiesis. Although unexplored experimentally, this hypothesis is supported by previous studies which showed that administration of live *Escherichia coli* or a heat-killed fraction (both containing NOD1L) is sufficient to rescue antibiotic-depleted hematopoiesis.[10] A variety of prevalent gut commensals, including the predominant *Bacteroidetes* and *Proteobacteria* phyla, produce NOD1L. The fact that such a ubiquitous microbial compound has potent effects on hematopoiesis may benefit the host, as diverse gut compositions would likely be able to maintain type I IFN–dependent hematopoiesis.

Another microbially derived metabolite, DAT, also promotes granulopoiesis in the absence of the microbiota, suggesting that diverse microbial compounds may act in concert to promote homeostatic hematopoiesis. However, DAT treatment was insufficient to rescue bone marrow LSK cells, and it remains unclear if the doses of DAT used in our study were insufficient to induce type I IFN signaling and rescue hematopoiesis or if DAT does not adequately traffic to the bone marrow. Nevertheless, we speculate that a variety of microbiota-derived metabolites may contribute to steady-state hematopoiesis. This model would explain why antibiotics with diverse spectra affect hematopoiesis in humans. Because each individual may depend on a unique set of bacterial taxa to provide the stimulus for basal type I IFN signaling, every person may have distinct vulnerabilities when treated with antibiotics of varying classes. The identification of other metabolites in the nontargeted metabolomics screen that can stimulate type I IFN signaling in hematopoietic progenitor cells supports this theory. Further work using defined communities of bacteria and combinations of antibiotics will be necessary to determine the extent to which specific taxa contribute to the overall phenotype of microbiota-driven steady-state hematopoiesis.

We identified microbial factors that may not only ameliorate cytopenias when given during antibiotic administration but also could have important implications for the field of bone marrow transplantation recovery. Given that the intestinal microbiota affects outcomes of bone marrow transplantation, microbial factors such as NOD1L that can promote various stages of hematopoiesis (Figure 3) may potentially improve clinical outcomes from transplantation. In addition, further examination of metabolites identified in our untargeted metabolomics screen may add to the number of therapeutic tools that could be used in this manner. Additional studies into hematopoiesis-supporting metabolites in humans are warranted and are the subject of ongoing research. Exploration of host and microbial factors that promote hematopoiesis will increase our understanding of how to mitigate the negative effects of antibiotic-induced loss of microbiota diversity on hematopoiesis without the need to cease antibiotic treatment.

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Authorship

Contribution: H.Y., F.C.W., M.T.B., and K.Y.K. designed experiments and wrote the manuscript; H.Y., F.C.W., A.A., and H.H. performed experiments and analyzed and interpreted results; and H.Y., K.Y.K., L.T., L.V., and P.L.L. designed the nontargeted metabolomics, performed metabolomics, and analyzed and interpreted metabolomics results.

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